Single residue within the antigen translocation complex TAP controls the epitope repertoire by stabilizing a receptive conformation

Christoph Baldauf*,1, Susanne Schrödt*1, Meike Herget*1, Joachim Koch*4, and Robert Tampé*6,6,2

*Institute of Biochemistry, †Center for Membrane Proteomics, and ‡Cluster of Excellence Frankfurt—Macromolecular Complexes, Goethe-University Frankfurt, Max-von-Laue-Strasse 9, D-60438 Frankfurt/Main, Germany

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The recognition of virus infected or malignantly transformed cells by cytotoxic T lymphocytes critically depends on the transporter associated with antigen processing (TAP), which delivers peptides to the endoplasmic reticulum lumen for subsequent loading of major histocompatibility complex class I molecules. Here we have identified a single cysteine residue in the TAP complex that modulates peptide binding and translocation, thereby restricting the epitope repertoire. Cysteine 213 in human TAP2 was found to be part of a newly uncovered substrate-binding site crucial for peptide recognition. This residue contacts the peptide in the binding pocket in an orientated manner. The translocation complex can be reversibly inactivated by thiol modification of this cysteiny1 residue. As part of an unexpected mechanism, this residue is crucial in complementing the binding pocket for a given subset of epitopes as well as in maintaining a substrate-receptive conformation of the translocation complex.

Results

Cys-Less TAP Displays an Altered Substrate Specificity. To investigate the impact of the 10 and 9 cysteines in TAP1 and TAP2, respectively, we combined the Cys-less (CL) and wild-type (WT) subunits and analyzed the effect of peptide recognition and ER translocation. TAP-specific and ATP-dependent peptide transport was examined by incubating microsomes with the fluorescently labeled, high-affinity epitope RRYQNSTC12-15/L (R9L-F) for 3 min at 32 °C. N-core glycosylated, thus translocated, peptides were recovered on ConA beads, eluted with methyl-α-D-mannopyranoside, and quantified. Notably, all constructs showed a TAP-specific, ATP-dependent peptide transport activity (Fig. L4). Each subunit was expressed at the same level as shown by immunoblotting. Slight changes in electrophoretic mobility of the TAP variants are due to a C-terminal His10 and Strep-tag of TAP1 and TAP2, respectively. Based on the epitope R10T-A (RYWANATRC12-15/L labeled with ATTO565), which was transported with the same efficiency by all complexes (gray bars) and therefore serves as reference, we surprisingly found that the CL/CL or WT/CL TAP1/2 complexes displayed a significantly lower transport activity for the epitope R9L-F compared to WT/WT or CL/WT (black bars). We conclude that the Cys-less TAP complex is functional with respect to peptide transport, however, displaying a different substrate specificity, which is caused by Cys-less TAP2.

Peptide translocation into the ER lumen is a multistep process, accompanied by structural rearrangements in the TMDs and NBDs (16, 17). We therefore examined if the altered substrate specificity is caused by a different binding mode. TAP-containing TAP1 or TAP2 can restore MHC I antigen presentation in TAP1- or TAP2-negative cells (15). However, functional details of a Cys-less TAP complex have not been investigated.

Here we have identified a single residue in the TAP2 subunit (Cys 213) that is crucial for recognition of a subset of peptides by the TAP complex. This residue interacts directly with the peptide in a defined orientation within the binding pocket. As part of an unexpected mechanism, Cys 213 is crucial to complement the substrate-binding pocket of the TAP complex by stabilizing a peptide-receptive conformation.

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1C.B. and S.S. contributed equally to this work.

2To whom correspondence should be addressed. E-mail: tampe@em.uni-frankfurt.de.

3Present address: Stanford University School of Medicine, Campus Drive, Stanford, CA 94305.

4Present address: Georg-Speyer-Haus, Institute of Biomedical Research, Paul-Ehrlich-Strasse 42-44, D-60596 Frankfurt/Main, Germany.

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Cysteines located outside of the previously identified peptide-binding region of TAP2 have a key role in modulating the substrate specificity of the TAP complex.

Single Cysteinyl Residue Modulates TAP Specificity. The peptide-binding region of TAP2 has been previously mapped to residues 330–452 (10). Notably, 3 out of 10 of the exchanged cysteines (C353, C362, and C394) are located within this region and thus might be critical for peptide binding. To address the impact of these residues, we reintroduced single cysteines at position 353, 362, and 394 in Cys-less TAP2 and combined those with Cys-less TAP1. Strikingly, this triple mutations of these three cysteines display an identical functional fingerprint as Cys-less TAP. These results together indicate that one or more of the 10 intrinsic cysteines of TAP2 play a key role in modulating the substrate specificity of the TAP complex.

Microsomes were incubated with the two radiolabeled epitopes on ice. In case of the epitope R9L, a drastically reduced binding activity was observed for the CL/CL or WT/CL complex in comparison with WT/WT or CL/WT (Fig. 1A, Lower), while binding of the epitope R10T is not affected (Table 1). These data demonstrate that one or more of the 10 intrinsic cysteines of TAP2 play a key role in modulating the substrate specificity of the TAP complex.

Table 1. Peptide dissociation constants $K_d$ and maximal binding $B_{\text{max}}$ (Eq. 51) of TAP variants for the different epitopes R9L and *R10T (in bold)

<table>
<thead>
<tr>
<th>TAP1/TAP2</th>
<th>$B_{\text{max}}$, %</th>
<th>$K_d$, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/WT</td>
<td>100.0 ± 5.1</td>
<td>493 ± 78</td>
</tr>
<tr>
<td>CL/CL</td>
<td>8.0 ± 1.5</td>
<td>688 ± 371</td>
</tr>
<tr>
<td>CL/CL(C197)</td>
<td>3.7 ± 0.8</td>
<td>861 ± 516</td>
</tr>
<tr>
<td>CL/CL(C209)</td>
<td>7.6 ± 1.1</td>
<td>913 ± 350</td>
</tr>
<tr>
<td>CL/CL(C213)</td>
<td>130.7 ± 6.9</td>
<td>536 ± 85</td>
</tr>
<tr>
<td>WT/WT*</td>
<td>100.0 ± 5.1</td>
<td>1,578 ± 450</td>
</tr>
<tr>
<td>CL/CL*</td>
<td>85.0 ± 6.5</td>
<td>1,700 ± 400</td>
</tr>
</tbody>
</table>

Cysteine 213 of TAP2 is Directly Involved in Substrate Binding. To investigate whether C213 of TAP2 is localized in the peptide-binding pocket, we performed cysteine cross-linking experiments with the single-cysteine CL/CL(C213) complex. Single-cysteine peptides can form a disulfide crosslink in the presence of copper phenanthroline only if two cysteines are in very close proximity. After quenching of free cysteines by N-ethylmaleimide (NEM), TAP complexes were purified and analyzed by nonreducing SDS-PAGE and autoradiography. Cross-linking was observed for peptides containing a cysteine at positions 4–9 (Fig. 2A). Notably, an excess of competitor peptide blocks the cross-linking, confirming the specificity of the reaction. These results demonstrate that C213 of TAP2 is in direct contact with the bound peptide and therefore part of the substrate-binding pocket of the TAP complex. Because the peptide positions 1 and 2 are not cross-linked, we conclude that the peptide is bound to TAP in an oriented fashion.

We next probed the functional importance of C213 by different thiol-specific reagents. As shown in Fig. 2B, modification of C213 blocks peptide binding to TAP. This effect is most likely caused by steric hindrance, because it is independent of the chemical properties of the reagent. The IC$_{50}$ of (2sulfonatoethyl)methanethiosulfonate (MTSES) was determined to be $52 ± 7 \mu M$ (Fig. 2C), suggesting a quantitative labeling at 10 mM of MTSES. Importantly, the binding activity of TAP could be fully restored by

Fig. 1. Function of cysteines in the human TAP complex. (A) Wild-type and Cys-less TAP subunits have a different impact on peptide binding and translocation. ATP-dependent transport assays were performed with microsomes (normalized to TAP2 expression) for 3 min at 32°C using 1 μM of R9L-F (RRYQNSTM/L, black bars) and R10T-A (RYWINATRCL, gray bars). After lysis, N-core glycosylated peptides were bound to ConA beads, eluted with methyl-D-mannopyranoside, and quantified by fluorescence detection. Transport of R9L-F by wild-type TAP was set to 100%. TAP-containing microsomes (20 μg protein per lane) were analyzed by SDS-PAGE (10%) followed by immunoblotting against TAP1 and TAP2 (mAb148.3 and mAb435.3, respectively). Peptide-binding studies were performed with microsomes (normalized to TAP2 expression) and 1 μM radiolabeled RR(125I)YQKSTEL for 20 min on ice and corrected for background binding. Peptide binding of wild-type TAP was set to 100%. All experiments were performed as triplicates. (B) C213 in TAP2 is critical for substrate binding and transport. Transport of R9L-F in the presence (black bar) or absence of MgATP (3 mM, open bar) was carried as described in A. Expression of and peptide binding to TAP mutants were analyzed as described in A. R9L-F binding of wild-type TAP was set to 100%. All experiments were performed as triplicates.
reductive cleavage with β-mercaptoethanol (β-ME) (Fig. 2D). In addition, methanethiosulfonate (MTS) reagents did not affect peptide binding to the CL/CL complex, demonstrating that the effects are specific for C213. Quantitative modification of C213 by MTSES was further demonstrated by subsequent labeling with 5-iodoacetamidofluorescein (5-IAF) and in gel fluorescence detection (Fig. 2E). For reference, 5-IAF did not label the Cys-less TAP complex. These results demonstrate that exclusively C213 is modified and that the microsome preparations are not contaminated by wild-type TAP. In summary, C213 in TAP2 is part of a newly identified binding pocket, in which peptides are fixed in a defined orientation. Thiol-specific modifications prove that this cysteinyl residue is critical for TAP function.

Modulation of the Binding Motif. After the important finding that a single residue within TAP2 controls the binding characteristics of the heterodimeric transporter, we next raised the question whether the C213-modulated epitope selection is based on differences in either the sequence or the length of the peptide. Therefore, we set up a competition assay using the permissive epitope R10T as reporter and peptide libraries of different length as competitors (Fig. 3A). As summarized in Fig. 3B, Cys-less and wild-type TAP have the same affinity toward the 9-, 10-, and 12mer library (IC_{50} ∼ 10 μM), demonstrating that the epitopes R9L and R10T are not differentiated with respect to their length. It is well established that the three N-terminal and the last C-terminal peptide residues are critical for TAP binding, whereas residues in between do not significantly contribute (12–14).

Fig. 2. Functional importance of the C213 in TAP2. (A) Oxidative cross-linking of CL/CL(C213) in microsomes (0.5 mg of total protein) with radiolabeled peptides containing a single cysteine (1.25 μM, C2 = RCYQKSTEL). Cross-linking was induced by adding 1 mM of copper phenanthroline in the presence or absence of the competitor RRYQKSTEL (0.25 mM). After metal affinity purification of the TAP complex, cross-linked products were analyzed by nonreducing SDS-PAGE (10%) and autoradiography. The TAP expression was confirmed by SDS-PAGE and immunoblotting against TAP1 and TAP2 (mAb148.3 and mAb435.3, respectively). The asterisk indicates a TAP1 degradation product. (B) Modification of C213 blocks peptide binding of TAP. CL/CL(C213) containing microsomes (20 μg of total protein) were incubated with various thiol-specific reagents [0.5 mM NEM; 10 mM MTSES; 1 mM [2-(trimethylammonium)ethyl]methanethiosulfonate; 2.5 mM (2-aminoethyl)methanethiosulfonate; 0.25 mM fluorescein-5-maleimide (F-Mal); 0.25 mM 5-iodoacetamidofluorescein (5-IAF)] for 15 min on ice. Subsequently, peptide-binding assays were performed using radiolabeled RR_{(125)}YQKSTEL (1 μM) as a reporter. (C) Inhibition of peptide binding by MTSES. To determine the half-maximum inhibition value (IC_{50}) of MTSES, CL/CL(C213) containing microsomes were incubated with an increasing concentration of MTSES followed by peptide-binding studies under conditions as described in Fig. 2A. The IC_{50} was determined to be 52 ± 7 μM (Eq. S2). (D) Reversible TAP inhibition by thiol-specific modification. CL/CL(C213) containing microsomes (20 μg of total protein) were incubated with 10 mM of MTSES for 15 min on ice and subsequently incubated with or without β-ME (100 mM) for 30 min on ice. Peptide binding was assayed with 1 μM of radiolabeled RR_{(125)}YQKSTEL. (E) Efficiency of MTS labeling was determined by means of accessibility for alkylation by S-IAF. After MTS labeling, samples were incubated with S-IAF (250 μM) for 15 min on ice and the relative amounts of 5-IAF-modified TAP were determined by in-gel fluorescence. To determine the maximal labeling capacity by S-IAF (shown as 100%), TAP was denatured by SDS (2%) for 20 min at room temperature and then labeled with 250 μM of S-IAF for 3 min before SDS-PAGE (10%) and immunoblotting. Asterisks indicate nonspecific labeling.
Therefore, we applied a scanning approach of combinatorial pep-tide libraries focusing on positions 1, 2, 3, and 9. The radiolabeled peptide R10T was used as reporter. The randomized peptide library (X9) served as internal reference, yielding 50% inhibition of peptide binding (IC50) at the given concentration (10 μM). Favored and disfavored sublibraries (i.e., MX9, XOX9, X9OX9, and X9O) are marked by a value below or above 50% binding, respectively. As summarized in Fig. 3 C–F, wild-type and Cys-less TAP have a very different binding motif. Although a negatively charged residue at positions 1, 2, and 3 is highly disfavored for the wild-type TAP, Asp is well accepted by the Cys-less complex. Similarly, bulky hydrophobic residues (Phe, Trp) at positions 1 and 2 are disfavored by wild-type, but preferred by Cys-less TAP As previously described, epitopes containing proline at position 2 are not recognized by human TAP (12, 13). Surprisingly, Cys-less TAP can accept these peptides. Differences for the C-terminal residue are even more pronounced. Although wild-type TAP has a marked preference for peptides harboring a basic or hydrophobic residue at their C terminus (12, 13, 18), Cys-less TAP is rather promiscuous in this position. Taken together, Cys-less TAP has an altered mode of binding. Compared to wild type, Cys-less TAP is more promiscuous toward the C-terminal residue of the bound peptide and shows a high binding affinity for peptides containing negatively charged residues at the N-terminal positions or aromatic residues at the second position.

Cysteine 213 Promotes a Peptide-Receptive Conformation. Based on the important finding that the altered binding motif of Cys-less TAP could be allocated to a single residue, we sought to understand the mechanistic details of this effect. Therefore, we compared the binding activities of wild-type, single-Cys, and Cys-less TAP complexes toward the epitope R9L and R10T . In the range of error, the CL/CL(C213) and WT complex displayed similar Kd and Bmax values (Table 1). These results were somewhat expected because the initial transport rates of the CL/CL(C213) complex is comparable to wild-type TAP (see Fig. 1B). By contrast, the Bmax values of the CL/CL, CL/CL(C209), and CL/CL(C197) complex are drastically reduced, whereas the Kd values do not differ significantly. Thus, only a limited fraction of the transport complexes is receptive for the epitope R9L. In contrast, the wild-type and Cys-less complexes show very similar Kd and Bmax values for the R10T epitope (Table 1). In conclusion, peptide binding and transport by TAP can be promoted either by the optimal peptide (R10T vs. R9L) or by C213, thus stabilizing a peptide-receptive conformation.

To corroborate this model, we developed a dual-color transloca-tion assay, which allowed us to follow the translocation rate of two epitopes in parallel (Fig. 4A). Peptides R9L-F and R10T-A were labeled with different fluorophores, with no spectral overlap. Notably, the fluorophores attached do not change the peptide-binding affinity to TAP. For R9L-F, the transport activity of Cys-less TAP is drastically reduced in comparison to wild type, whereas R10T-A is translocated equally well by both complexes, confirming our previous results. We next examined the peptide RRYQNSTEL (R9L) and EPYGTNSTD (E9D), which are high-affinity and nonbinder to wild-type TAP, respectively (13). If R9L is present in equimolar ratio, the transport activity of R9L-F by wild-type and Cys-less TAP is reduced by 50% (black bars). In contrast, the transport activity of R10T-A by wild-type TAP is decreased to 50% as expected, whereas the Cys-less complex has 75% remaining transport activity in the presence of the competitor R9L (open bars). This underscores the existence of R9L-receptive and nonreceptive CL/CL complexes. These findings are finally confirmed by dual-color transport assays. For wild-type TAP, the peptide transport activity is similar (~50%) for both peptides, whereas for Cys-less TAP, one population transports 50% of R10T, and R9L is transported only by a subpopulation of transporters (~20%). For wild-type and the R9L-receptive TAP complexes, transport of R9L-F was not affected by the highly disfavored epitope E9D. In contrast, transport of R10T-A by Cys-less TAP was inhibited by E9D, demonstrating that the specificities of the wild-type and Cys-less complexes are drastically different. These results provide further evidence for a receptive and nonreceptive conformation of the Cys-less complex for a given subset of peptides.

Discussion

As the key component of the MHC I peptide-loading complex, TAP translocates a smidgen of the cellular proteome into the ER lumen for the processive assembly of MHC I molecules. Cys-less TAP has been instrumental to determine the membrane topology of the TAP complex by membrane impermeable thiol-specific probes and cysteine scanning approaches (15). Cys-less TAP and TAP2 can restore antigen processing and MHC I surface expression in cells lacking either TAP1 or TAP2 (19). However, based on the normalization of the TAP level by immunoblotting and binding assays (Bmax value) using the radiolabeled peptide RR(125I)YWANATRST, the surprising effects on altered epitope selection and transport could not be detected in previous studies. However, (i) by direct comparison of different epitopes, RRYQNSTC(F)EL vs. RYYWANACR(T)A/T, (ii) by generating a systematic set of TAP mutants, (iii) by the use of combinatorial peptide libraries, and (iv) by establishing a dual-transport assay to follow the translocation of different epitopes in parallel, the
appealing differences of the wild-type and Cys-less TAP complex became obvious. By comparison of the binding and transport characteristics of wild-type and Cys-less chimera, we could identify Cys 213 in TAP2 as a critical residue modulating the substrate specificity of the TAP complex. This finding is instrumental to our current understanding of solute translocation across cellular membranes. Apart from defects in misfolding and/or trafficking, mutation of a single residue may effect conformational changes as shown for the lactose permease LacY, a member of the major facilitator superfamily. In this case, the mutation C154G results in a conformational arrest of the transporter, which was essential for crystallization and structure determination of this transporter (20, 21).

The functional importance of C213 within TAP2 was investigated by means of thiol-reactive probes. Modifications of C213 by charged or hydrophobic probes resulted in an inactive TAP complex, reflecting the importance of the free thiol side chain for peptide binding and translocation. Importantly, the inhibition of the TAP complex is reversible as demonstrated by the reductive cleavage of disulfide bonds by hydrophobic reducing reagents (β-ME). More hydrophilic reducing agents, such as DTT, were not able to fully restore TAP activity despite its higher reducing potential, indicating a more hydrophobic environment of this cysteinyl residue. Because modification of C213 results in a reversible switch between an active and inactive translocation complex, we speculated whether this effect is due to a conformational arrest. Reminiscent to these findings, charge-altering mutations at Arg 352 destabilize the open conformation of the cystic fibrosis transmembrane conductance regulator and also alter the ion selectivity filter (22).

Interestingly, the dissociation constant ($K_d$) of the epitope R9L for Cys-less TAP is in the same range as for the wild-type complex, but the number of binding sites ($B_{\text{max}}$ value) for CL/CL are drastically reduced. However, for the epitope R10T, Cys-less and wild-type TAP show the same mode of peptide binding and transport. Taken together, these findings exclude a defect in folding and propose a model favoring a peptide-receptive state. This may correspond to an outward-facing or occluded conformation as observed in lactose permease or ABC exporters Sav1866 and MsbA (23–25). Similarly, a mutation in lactose permease (C154G) results in an opening of a hydrophilic pathway to the periplasmic side (20). In case of the yeast multidrug transporter Pdr5, it has been proposed that the kinetics of the translocation cycle of the ABC transporter dictate substrate selection (26). However, in contrast to Pdr5, the ATPase activity of the TAP complex is strictly coupled to substrate binding (27, 28). Here we demonstrate that the initial translocation rate of Cys-less and wild-type TAP do not differ, thus excluding the possibility that kinetic effect of the transport cycle gives rise to this unusual behavior.

Cross-linking studies with the CL/CL(C213) complex revealed that the peptide is oriented in the binding pocket with the C-terminal half pointing toward C213 of TAP2. Remarkably, for the epitope R10T, wild-type and Cys-less TAP display identical binding and transport characteristics. Hence a nonreceptive conformation of CL/CL can be unlocked and fully populated by a subset of epitopes. Competition assays with peptide libraries revealed

![Fig. 4. Dual-color translocation assays reveal restrictions in a shared TAP1/2 interface.](image)
that wild-type and Cys-less TAP have a distinct but overlapping binding motif. The differences are spotted to the three N-terminal and C-terminal residues of the bound peptide, whereas the peptide length is not critical. In contrast to wild type, the Cys-less complex can accept negatively charged residues at positions 1 and 2 and, in particular, at the C terminus. Mutating cysteine 213 to either alanine or serine may abrogate the electrostatic effect between the thiolate and negatively charged residues of the bound peptide. This may also reflect the local environment, which causes a deprotonation of the sulfhydryl group (pH < pKₐ). Collectively, these data demonstrate that the cysteine residue 213 in TAP2 is crucial for peptide recognition of the antigen translocation machinery. Cys-less and wild-type TAP recognize distinct, but overlapping, sets of peptides.

Based on a homology model derived from the X-ray structure of the ABC exporter Sav1866 (25, 29), C213 is located at the membrane/cytosol interface at the beginning of the cytosolic loop 2 of core TAP2, whereas C197 and C209 are embedded in the ER membrane as part of TM2 (Fig. 4 B and C). Residues 217 (Thr) and 218 (Met), as well as 374 (Ala) and 380 (Arg) of TAP2 have been identified to control the peptide repertoire (30–32). Interestingly, residues 213, 217, 218, and 374 as well as the membrane/cytosol interface at the beginning of the cytosolic loop 2 are conserved across mammalian TAP2 sequences.

In summary, C213 of TAP2 is crucial in maintaining a peptide-binding conformation by providing additional contact sites to orient the peptide in the binding pocket. Although, based on an altered binding motif, a subset of epitopes can compensate the lack of C213, this essential residue stabilizes a peptide-receptive conformation and represents a critical element for epitope selection in the pathway of MHC class I antigen presentation.

Materials and Methods

Details of materials, cloning, expression, membrane preparation, and peptide-binding assays are provided in *St Text.*

Cloning and expression. Based on human tap1 and tap2, combinations of Cys-less (CL) and wild-type (WT) TAP subunits were generated. The tap1CL gene was cloned into the BamHI and HindIII sites of pFastBacDual™ (Invitrogen) downstream of the polyhedrin promoter and combined with tap2. The tap2 was cut from p46TAP2WT via NotI (treated with Klenow) and NsiI and cloned into pFastBacDual.TAP1CL via XhoI (treated with Klenow) and NsiI (5). The resulting plasmid was termed pFastBacDual.TAP1CL/TAP2WT. The combination of tap1 and tap2 was generated by cloning tap1CL into the pFastBacDual.TAP1WT plasmid via XhoI and NsiI. The resulting plasmid was termed pFastBacDual.TAP1WT/TAP2CL.

To generate a TAP complex containing cysteine residues only in the N-terminal region (1-265) of TAP2, first a 5′ XhoI/DraI fragment of tap2 was combined in a single ligation step with a 3′ DraI/NsiI fragment of tap2CL in pFastBacDual.TAP1CL, which was opened with XhoI and NsiI. The resulting plasmid was termed pFastBacDual.TAPCL/TAP2CL(1-265WT). Single cysteines were reintroduced into tap2CL at the original position by site-directed mutagenesis to generate CL/single-Cys complexes. The following sense oligonucleotides were used to introduce cysteines at the indicated positions: TAP2A197C, 5′-ATGCTGGTCC-3′; TAP2S213C, 5′-GCGGTTGCTGGCAAC-3′; TAP2V394C-3′; TAP2S362C, 5′-GAGGCGTGCTGCAAC-3′ (exchange nucleotides are underlined). All constructs were confirmed by sequencing. Single-Cys constructs of tap2 were combined with tap1CL, resulting in plasmids encoding always both subunits (pFastBacDual.TAP1CL/TAP2CL/single-Cys).

Recombinant baculovirus and cell culture. Bacmid DNA was produced by transformation of DH10Bac cells with pFastBacDual-TAP (Invitrogen). Insect cells (Spodoptera frugiperda, Sf9) were grown in Sf900II medium (Invitrogen) following standard procedures. Recombinant baculovirus was generated and used for infection of insect cells as described previously (4).

Membrane preparation. For crude membrane preparation, 2.0 × 10⁶ insect cells per milliliter were resuspended in Tris-buffer (10 mM Tris · HCl, 1 mM DTT; pH 7.4) and homogenized with a tight glass Dounce homogenizer (Wheaton). Nuclei and cell debris were removed by centrifugation at 200 × g for 4 min followed by 700 × g for 8 min at 4°C. Membranes were harvested at 100,000 × g for 30 min at 4°C, washed, and resuspended in PBS (pH 7.4) to a final concentration of 5 mg protein per milliliter. Aliquots were frozen in liquid nitrogen and stored at −80°C. All buffers used for preparation were supplemented with protease inhibitors [50 μg/mL 4-(2-aminoethyl)benzenesulfonyle fluoride, 1 μg/mL aprotinin, 150 μg/mL benzamidine, 10 μg/mL leupeptin, 5 μg/mL pepstatin]. The preparation of microsomes was performed as described previously (1).

Peptide binding. Peptides were radiolabeled with Na[125I] using the chloroform T method (6). Peptide binding to TAP was performed using filter assays. Filter plates (MultiScreen plates with a glass fiber filter, pore size 1 μm; Millipore) were preincubated with 0.3% polyethyleneimine. TAP-containing membranes (20 μg of total protein) were incubated with 1 μM or with various concentrations of radiolabeled peptides (RRYQKSTEL or RYWA-NATRSX, where X represents Phe, Lys, Val, or Thr) in 50 μL of binding buffer (PBS with 5 mM MgCl₂; pH 7.0) for 20 min on ice. Subsequently, the membranes were transferred to filter plates and washed twice with 100 μL of ice-cold binding buffer. Filter-associated radioactivity was quantified by γ-counting. Unspecific binding was determined in the presence of a 400-fold molar excess of unlabeled peptide (RRYQKSTEL) or in the presence of the TAP specific Herpes Simplex viral inhibitor ICP47 (50 μM) (7). Specifically bound peptides were plotted against the total peptide concentration and fitted to a Langmuir (1:1) binding model:

\[ B = \frac{B_{\text{max}} \times [C]}{K_D + [C]}, \]  

where \( B \) represents the bound peptide, \( B_{\text{max}} \) the maximal amount of bound peptide, \( [C] \) the peptide concentration, and \( K_D \) the dissociation constant, respectively.

The affinities of the peptide libraries were determined by a competition assays in order to determine in the half-maximal inhibitory concentration (IC₅₀). TAP-containing membranes (20 μg of protein) were incubated with 1 μM of the radiolabeled peptide RWYANATRS and various concentrations of the peptide library and treated as described above. To calculate the IC₅₀, the amount of specifically bound peptides was plotted against the peptide concentration and fitted with the following equation:

\[ B = \frac{B_{\text{max}} - B_{\text{min}}}{1 + \frac{10^{[C]/L}}{K_D}}, \]  

where \( B \) corresponds to the bound peptide, \( B_{\text{max}}/B_{\text{min}} \) to the amount of bound peptide (maximal and minimal), \( [C] \) to the peptide concentration, and IC₅₀ to the half-maximal inhibitor concentration, respectively.

For TAP inhibition, microsomes were incubated with NEM (500 μM), FM (250 μM), 5-IAF (250 μM), 2-[2-(trimethylammonio)ethyl]methanesulfonate bromide (1 mM), (2-sulfonatoethyl) methanesulfonate (10 mM), or (2-aminoethyl) methanesulfonate (2.5 mM), respectively, for 15 min at 4°C (8). After washing with PBS, peptide binding was measured using 1 μM of radiolabeled peptide as described above. To reverse MTS labeling, samples were incubated with 100 mM of β-mercaptoethanol (β-ME) for 30 min on ice. After washing with PBS, peptide-binding assays were performed as described above. The MTS-labeling efficiency was determined via 5-IAF alkylation (250 μM) for 15 min at 4°C in the dark. Samples were washed twice prior to the analysis by SDS-PAGE (10%) and immunoblotting.
Relative amounts of 5-IAF-labeled TAP were determined by in-gel fluorescence with a Lumi Imager F1 (Roche). In order to determine the maximal labeling capacity, TAP was denatured with 2% SDS for 20 min at room temperature and then labeled with 250 μM of 5-IAF for 3 min prior to the analysis by SDS-PAGE (10%) and immunoblotting.